

FLICE, A Novel FADD-Homologous ICE/CED-3-like Protease, Is Recruited to the CD95 (Fas/APO-1) Death-Inducing Signaling Complex

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Summary

To identify CAP3 and CAP4, components of the CD95 (Fas/APO-1) death-inducing signaling complex, we utilized nano-electrospray tandem mass spectrometry, a recently developed technique to sequence femtomole quantities of polyacrylamide gel-separated proteins. Interestingly, CAP4 encodes a novel 55 kDa protein, designated FLICE, which has homology to both FADD and the ICE/CED-3 family of cysteine proteases. FLICE binds to the death effector domain of FADD and upon overexpression induces apoptosis that is blocked by the ICE family inhibitors, CrmA and z-VAD-fmk. CAP3 was identified as the FLICE prodomain which likely remains bound to the receptor after proteolytic activation. Taken together, this is unique biochemical evidence to link a death receptor physically to the proapoptotic proteases of the ICE/CED-3 family.

Introduction

Apoptosis, or programmed cell death, is a genetically regulated mechanism with a central role in both metazoan development and homeostasis (Raff, 1992; Steller, 1995). The cell death machinery is conserved throughout evolution (Vaux et al., 1994) and is composed of several distinct parts, including effectors, inhibitors, and activators (Chinnaiyan and Dixit, 1996; Steller, 1995). Invertebrate model systems have been invaluable in identifying and characterizing the genes that control apoptosis (Hengartner, 1996). While numerous candidate genes

have been identified, the way in which they interact to execute the apoptotic program is poorly understood.

It is becoming apparent that cysteine proteases related to the *Caenorhabditis elegans* cell death gene *ced-3* represent the effector components of the apoptotic machinery. The first mammalian homolog of CED-3 identified was interleukin-1 β -converting enzyme (ICE; Yuan et al., 1993). Overexpression of ICE or CED-3 in Rat-1 fibroblasts induced apoptosis, suggesting that ICE was functionally, as well as structurally, related to CED-3 (Miura et al., 1993). However, such evidence is only a correlation, as ectopic expression of a number of proteases, including chymotrypsin, proteinase K, and trypsin, cause significant apoptosis (Williams and Henkart, 1994).

Further studies suggest that proteases related to ICE, rather than ICE itself, may play a more important role in the apoptotic mechanism. First, a number of cell types stably secrete mature IL-1 β without undergoing apoptosis. Second, ICE deficient mice, although unable to generate active IL-1 β , fail to exhibit a prominent cell death-defective phenotype (Kuida et al., 1995; Li et al., 1995). Third, in an in vitro model of apoptosis, condemned phase extracts prepared from chicken DU249 cells fail to cleave the primary substrate of ICE, pro-IL-1 β (Lazebnik et al., 1994). Instead, a proteolytic activity in these extracts, termed pICE, cleaves the DNA repair enzyme poly (ADP-ribose) polymerase (PARP) into signature apoptotic fragments (Lazebnik et al., 1994). Purified ICE fails to cleave PARP (Lazebnik et al., 1994; Tewari et al., 1995), suggesting that pICE is distinct from ICE.

To date, seven homologs of CED-3 and ICE have been characterized, including Nedd-2/ICH-1 (Kumar et al., 1994; Wang et al., 1994), Yama/ CPP-32/ Apopain (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Tewari et al., 1995), Tx/ICH-2/ICE rel-II (Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995), ICE rel-III (Munday et al., 1995), Mch-2 (Fernandes-Alnemri et al., 1995a), ICE-LAP3/Mch-3/CMH-1 (Duan et al., 1996a; Fernandes-Alnemri et al., 1995b; Lippke et al., 1996), and ICE-LAP6 (Duan et al., 1996b). Ectopic expression of these ICE/CED-3 homologs in a variety of cells causes apoptosis. Only Yama and ICE-LAP3 have been shown to be proteolytically activated by apoptotic stimuli (Chinnaiyan et al., 1996a; Duan et al., 1996a; Schlegel et al., 1996). Future studies will delineate which family members have an important role in the apoptotic mechanism.

Although it is clear that CED-3-like proteases are distal effectors of the cell death pathway, the proximal components that mediate their activation remain to be identified. Two cell surface cytokine receptors, CD95 (Fas/APO-1) and TNFR-1, have been shown to trigger apoptosis by their natural ligands or specific agonist antibodies (Baglioni, 1992; Itoh et al., 1991; Trauth et al., 1989). Both death receptors are members of the tumor necrosis factor (TNF)/nerve growth factor receptor family, which also includes TNFR-2, low affinity NGFR, CD40, and CD30, among others (Smith et al., 1990; Tewari and Dixit, 1995). While family members

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are defined by the presence of cysteine-rich repeats in their extracellular domains, CD95 and TNFR-1 also share a region of homology, appropriately designated the "death domain," required to signal apoptosis (Itoh and Nagata, 1993; Tartaglia et al., 1993). This shared death domain suggests that both receptors interact with a related set of signal-transducing molecules that, until recently, remained unidentified. Using the two-hybrid system, three death domain-containing molecules, TNFR1-associated death domain (TRADD), Fas-associating protein with death domain (FADD)/MORT1, and receptor-interacting protein (RIP), have been isolated (Boldin et al., 1995; Chinnaiyan et al., 1995; Cleveland and Ihle, 1995; Hsu et al., 1995; Stanger et al., 1995). Subsequent studies show that endogenous FADD associates with CD95 in an activation-dependent fashion (Kischkel et al., 1995), while, similarly, endogenous TRADD and RIP have been found complexed to activated TNFR-1 (Hsu et al., 1996a, 1996b). It has been postulated that TRADD acts as an adaptor molecule for TNFR-1 (Hsu et al., 1996a), mediating the interaction of TNFR-1 with FADD, while, by contrast, RIP may be involved in NF- κ B signaling (Hsu et al., 1996b). A dominant negative version of FADD (FADD-DN) blocks TNF- and CD95-induced apoptosis, suggesting that FADD functions as the common signaling conduit for cytokine-mediated cell death (Chinnaiyan et al., 1996a; Hsu et al., 1996a).

The first evidence for the involvement of ICE-like proteases in CD95- and TNFR-1 signaling came with the discovery that the poxvirus gene product CrmA blocks cell death triggered by both receptors (Enari et al., 1995; Los et al., 1995; Tewari and Dixit, 1995b). In vitro, the serpin CrmA interacts only with the active forms of ICE and ICE-like proteases (Ray et al., 1992; Tewari et al., 1995). Yama and ICE-LAP3, two of the ICE-like enzymes most related to CED-3, are expressed as zymogens that are proteolytically activated upon ligation of CD95 or TNFR-1 (Chinnaiyan et al., 1996b; Duan et al., 1996a). However, both Yama and ICE-LAP3 remained as pro-zymogens in anti-CD95-treated CrmA-expressing cells, suggesting that CrmA inhibits an ICE-like protease upstream of Yama and ICE-LAP3 (Chinnaiyan et al., 1996b). The mechanism by which the death receptors engage the cytosolic apoptotic proteases is of central importance to cell death research.

Activation of CD95 initiates association with at least four proteins designated CAP, for cytotoxicity-dependent APO-1-associated proteins (Kischkel et al., 1995). CAP1 and CAP2 have been identified as alternate forms of serine-phosphorylated FADD, while the identity of the other CAPs, CAP3 and CAP4, remains to be determined. The four associating proteins, along with the oligomerized receptor, have been termed the CD95 death-inducing signaling complex (DISC; Kischkel et al., 1995). Later studies demonstrated that a dominant negative version of FADD, missing the N-terminal death effector domain (DED), functions by blocking the recruitment of CAP3 and CAP4 to the DISC (Chinnaiyan et al., 1996a). Thus, these results strongly suggest that CAP3 and CAP4 are downstream components of the CD95 signaling cascade.

Recently, the method of protein sequencing using

electrospray (Fenn et al., 1989), in combination with tandem mass spectrometry (Hunt et al., 1986), was refined to allow sequencing of femtomole quantities of proteins directly isolated from silver-stained gels (Wilm et al., 1996). The peptide sequence tags (Mann and Wilm, 1994) generated can then be used to screen sequence databases to obtain matching sequences leading to isolation of full-length clones for functional characterization. In its first reported use, an antiangiogenic factor derived from mycoplasma was characterized using this technique (Wilm et al., 1996).

To identify CAP3 and CAP4, we took advantage of the above technology of nano-electrospray tandem mass spectrometry (nano-ES MS/MS). CAP4 is a novel cysteine protease of the ICE/CED-3 family that contains a prodomain homologous to FADD and has therefore been designated FLICE (for FADD-like ICE). CAP3 was identified as the prodomain of FLICE likely generated during CD95-induced proteolytic activation. In vitro, the DED of FADD can directly bind FLICE. Thus, these results suggest that CD95 utilizes the adaptor protein FADD physically to engage FLICE, the apical component of a proteolytic cascade made up of other ICE/CED-3-like proteases.

Results

Identification of FLICE (CAP4/CAP3)

At least four endogenous proteins, CAP1-4, associate with activated CD95 to form the DISC (Kischkel et al., 1995). CAP1 and CAP2 have been identified as different forms of the previously isolated FADD/MORT1 (Kischkel et al., 1995). Subsequent dominant-negative studies have established that endogenous FADD is essential for the recruitment of CAP3 and CAP4 to the CD95 DISC (Chinnaiyan et al., 1996a). To identify CAP4, we utilized nano-ES MS/MS (Mann and Wilm, 1995; Wilm and Mann, 1996) to generate peptide sequence from gel-isolated protein (Figure 1). The CAP4 spot contained approximately 0.5 pmol of protein. The complete sequence of five peptides, covering a total of 41 amino acid residues and two partially sequenced peptides that could be used as sequence tags, was determined (Figure 2A). Homology searches against a comprehensive nonredundant database (NRDB, maintained by C. Sander, European Molecular Biology Laboratory) revealed no matches to known proteins. However, when the Human Genome Sciences cDNA database was searched, a 3.0 kb cDNA was identified and found to contain a 1437 bp open reading frame that encoded a novel protein with a predicted molecular mass of 55.3 kDa and a pI of 4.91, consistent with the size and pI of CAP4, as determined by 2D gel analysis (Figures 1A and 1B). The putative initiator methionine (AAGATGG) was in agreement with the consensus Kozak's sequence for translation initiation. A BLAST search of the GenBank protein database revealed that the novel cDNA, designated FLICE, had substantial homology to both FADD and the ICE/CED-3 family of cysteine proteases. CAP3 was also subjected to nano-ES MS/MS. Peptides (2) were sequenced, both being identical to two peptides (T2 and T4) found in the sequencing of the total tryptic digest of CAP4 (Figure

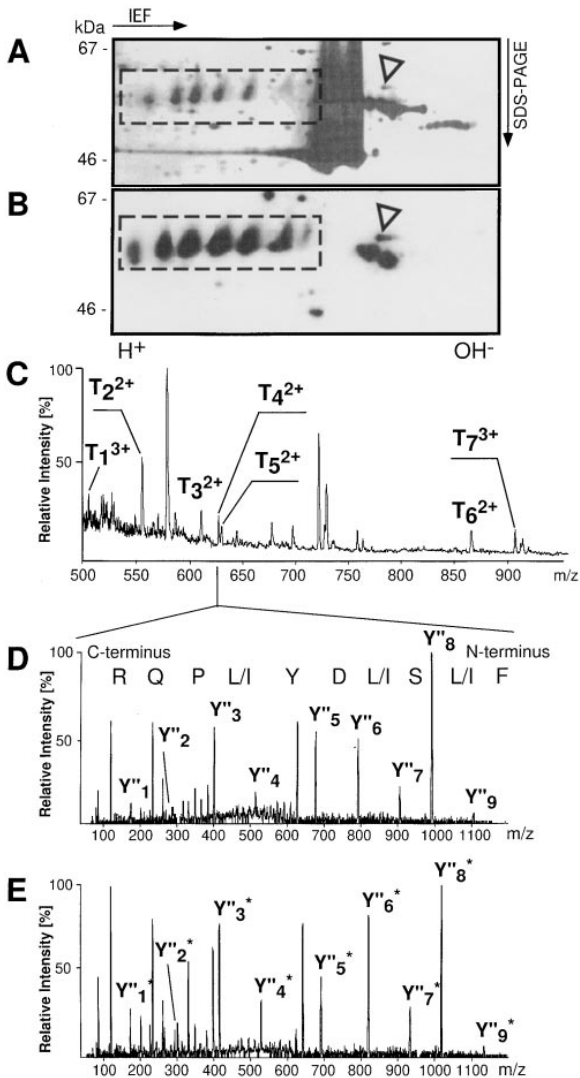


Figure 1. Nano-ES MS/MS Sequence of CAP4

(A) Preparation and sequence analysis of CAP4. Silver-stained 2D IEF/10% SDS-PAGE of immunoprecipitated DISC from K562 cells. (B) Autoradiography of an analysis of metabolically labeled DISC proteins separated on a 2D IEF/10% SDS-PAGE gel. Preparation of the ³⁵S-labeled DISC was done as previously described (Kischkel et al., 1995). Migration positions of CD95 spots in (A) and (B) are labeled by a stippled box. The migration position of CAP4 is indicated by an arrowhead.

(C) Part of the mass spectrum of the peptide mixture extracted after in-gel digestion of the CAP4 spot with trypsin. Peaks of tryptic peptides (T1-T7) were sequenced by nano-ES MS/MS. Most of the unlabeled peaks are trypsin autolysis products.

(D) Sequencing of peptide number 4 shown in C (m/z = 626.5) with tandem mass spectrometry. Fragmentation of tryptic peptides at the amide bonds predominantly produced ion series containing the C-terminus (designated Y''¹, Y''², etc.; Roepstorff and Fohlman, 1984). Half of the tryptic digest was analyzed in this experiment.

(E) Tandem mass spectrum of the same peptide after esterification of the second half of the total tryptic digest mixture (Hunt et al., 1986). Esterification resulted in a characteristic 14 kDa mass shift of the C-terminus containing the fragment ions (Y''ⁿ ions) and an additional shift of 14 kDa for each Asp and Glu residue. Software-assisted comparison of the two spectra in (D) and (E) allowed unambiguous assignment of peptide sequence. The following sequences were obtained: T1, LLNDYEEFSKER; T2, FLLQEELSK; T3, QMPQPT FTLR; T4, FLSDLYLPQR; T5, LLNDYEEFSK. Note that, due to the

2A). Therefore, CAP3 represents an N-terminal fragment of FLICE.

FLICE Has Homology to the DED of FADD

The association of CD95 with FADD is mediated by their respective C-terminal death domains (Boldin et al., 1995; Chinnaiyan et al., 1995). A truncated derivative of FADD (FADD-DN), which contains the death domain but lacks the N-terminus, functions as a potent dominant-negative regulator of CD95-induced apoptosis (Chinnaiyan et al., 1996a). By contrast, the 117 N-terminal amino acids of FADD are capable of triggering apoptosis, and thus, this segment has been termed the death effector domain (Chinnaiyan et al., 1995). Interestingly, FLICE contains two N-terminal stretches of approximately 60 amino acids that are homologous to the DED of FADD (Figures 2B and 3A). A BLAST search revealed that residues 7-75 and 101-169 of FLICE matched the DED of FADD (residues 4-76) and shared 39% identity (55% similarity) and 28% identity (55% similarity), respectively. Another protein, designated PEA-15, was also identified as a DED-containing protein by BLAST search. PEA-15 is an astrocytic phosphoprotein of unknown function (H. Cheneiweiss, unpublished data; GenBank accession number S55384).

FLICE Is a Novel Member of the ICE/CED-3 Family

While the N-terminus of FLICE contained the FADD homology domains, the remainder of the protein was highly homologous to the ICE/CED-3 family, particularly in the regions corresponding to the active subunits of ICE (Figure 3B). Phylogenetic analysis of the ICE/CED-3 gene family showed that FLICE was a member of the CED-3 subfamily that included Yama/ CPP-32, Mch-2, ICE-LAP3, and ICE-LAP6 (Figure 3C). Like *C. elegans* CED-3, FLICE contained a long N-terminal putative prodomain, but in this case, importantly, the prodomain shared homology to the DED of FADD. It is likely that CAP3 represents this prodomain, since its estimated molecular mass (26 kDa) by 2D gel analysis corresponds exactly with the calculated molecular mass of the putative FLICE prodomain (amino acids 1-220). There was no DED homology present in any of the other ICE/CED-3 family members.

Based on the X-ray crystal structure of ICE (Walker et al., 1994; Wilson et al., 1994), the amino acid residues His-237, Gly-238, and Cys-285 of ICE are involved in catalysis, while the residues Arg-179, Gln-283, and Arg-341 form a binding pocket for the carboxylate side chain of the P1 aspartic acid. These six residues are conserved in all ICE/CED-3 family members thus far cloned as well as in FLICE. However, residues that form the P2-P4 binding pockets are not widely conserved among family members, suggesting that they may determine substrate specificity. Interestingly, FLICE contained a unique pentapeptide, QACQG, instead of the QACRG

sequencing method, the isobaric amino acids isoleucine and leucine could not be distinguished. For peptides T6 and T7, peptide sequence tags were obtained (Mann and Wilm, 1994) which uniquely identified the peptides VFFIQACQGDNYQK and GIPVETDSEEQPYL EMDLSSPQTR, respectively, within the FLICE sequence.

A

MDFSRNLYDIGEQLDSEDLASLKFFLSLDYIPQRKQEPKDALMLFQRLQEKRMLE
 ESNLSFLKELLFRINRLDLLITYLNTRKEEMERELQTPGRAQISAYRVMLYQISE
 EVSRSELRSFKFLLOEEISKCKLDDDMNLLDIFIEMEKRVILGEGKLDILKRVCA
 QINKSLLKIINDYEEFSKERSSSLEGGSPDEFNNGEELCGVMTISDSPREQDSESQ
 TLDKVVYQMKSKPRGYCLIIINHNFAKAREKVKPLHSIRDRNGTHLDAGALTTFE
 ELHFEIKPHDDCTVEQIYEILKIYQLMDHSNMDCFICCLSHGDKGIIYGTGQE
 PPIYELTSQFTGLKCPSLAGKPKVFFIQACQGDNYQKGIPIVETDSEEPYLEMDDL
 SSSPQTRYIPDEADFLGMATVNNCVSYRNPAGETWYIQSLWQSLRERCPRGDDIL
 TILTEVNYEVSNKDDKKNMGRQMPQPTFLRKKLVFPSPD

B

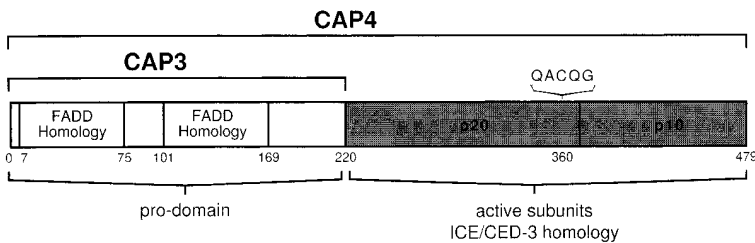


Figure 2. Sequence Analysis of FLICE

(A) Deduced amino acid sequence of the FLICE gene product. Sequences (T1-T7) obtained by nano-ES MS/MS are underlined. Peptide T5 was contained in the sequence of peptide T1. Peptides T6 and T7 (stippled line) were peptide sequence tags. The QACQG conserved pentapeptide containing the catalytic cysteine is indicated in bold.

(B) Schematic model of the FLICE protein. The N-terminal portion of FLICE (prodomain) contains two domains that show marked homology to the N-terminal DED domain of FADD (shaded boxes). The C-terminus of FLICE encodes a novel member of the ICE/CED-3 family of cysteine proteases (filled boxes). The pentapeptide QACQG is indicated.

shared by most other family members (Figure 3B), with the exception of the recently cloned ICE-LAP6, which contains a QACGG pentapeptide (Duan et al, 1996b).

Tissue Distribution of FLICE

Northern blot analysis revealed that FLICE was constitutively expressed in many fetal and adult human tissues, but not in the fetal brain (Figure 4). Interestingly, there was relatively higher expression of FLICE in peripheral blood leukocytes, which was consistent with the important role of CD95-signaling in lymphocyte homeostasis (Nagata and Golstein, 1995). The mRNA transcript was approximately 3.0 kb (Figure 4).

FLICE Associates with the DED of FADD

Previous studies show that FADD-DN (missing the DED) blocks the recruitment of CAP4 to the DISC, suggesting that the DED is an essential component (Chinnaiyan et al., 1996a). To determine whether FLICE can directly bind FADD and whether the DED of FADD is necessary for this binding, *in vitro* transcribed and translated FLICE was precipitated with His-6-tagged FADD and His-6-tagged FADD-DN immobilized onto Ni²⁺ beads. As predicted, FLICE bound full-length FADD but not FADD-DN (Figure 5A). Further confirmation was obtained by using 293T cell-generated proteins. In this case, 293T cells were transiently transfected with C-terminal-tagged FADD-AU1 and ΔFADD-AU1, in which the 18 N-terminal amino acids of the DED were deleted. Cell lysates were then immunoprecipitated with anti-AU1 antibody and protein G-coupled sepharose. The beads were subsequently incubated with ³⁵S-labeled FLICE or Yama/CPP-32, a related ICE/CED-3 family member. Consistent with results obtained in Figure 5A, FLICE, but not Yama, bound full-length FADD (Figure 5B).

Granzyme B-Activated FLICE Cleaves PARP

Members of the ICE/CED-3 gene family are synthesized as proenzymes and activated by proteolytic cleavage at specific aspartate residues to form heterodimeric enzymes. In ICE, this cleavage removes the prodomain and produces a heterodimeric complex consisting of p20 and p10 subunits (Thornberry et al., 1992). Similarly, activated Yama is comprised of two subunits, p17 and p12, which are derived from a 32 kDa proenzyme (Nicholson, 1996). Recent studies on granzyme B suggest that cytotoxic lymphocytes may utilize this secreted serine protease to activate directly members of the ICE/CED-3 family. It has previously been demonstrated that granzyme B can proteolytically activate pro-Yama, generating an active enzyme capable of cleaving the death substrate PARP into characteristic fragments (Darmon et al., 1995; Quan et al., 1996). By contrast, ICE, although cleaved by granzyme B, fails to be activated (Quan et al., 1996).

Thus, we determined whether FLICE could serve as a substrate for granzyme B and, more importantly, whether FLICE could function as a cysteine protease. *In vitro* transcribed and translated FLICE and Yama were incubated with purified granzyme B (Hanna et al., 1993). After 4 hr at 37°C, FLICE and Yama were proteolytically processed, generating putative active p20/p10 and p17/p12 subunits, respectively (Figure 6A). Next, we assessed whether granzyme B-mediated cleavage of FLICE generated an active enzyme by assaying for PARP cleavage. PARP is proteolyzed during many forms of apoptosis, and the enzyme or enzymes responsible likely belong to the ICE/CED-3 family (Nicholson et al., 1995; Tewari et al., 1995). To exclude the possibility of direct cleavage of PARP by granzyme B, granzyme B-processed FLICE and Yama were incubated with a

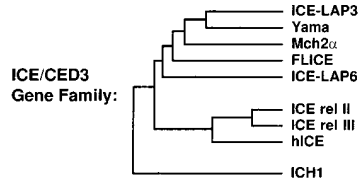
A

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FADD (1-35)  M D P P L V L L H S V S S S L S S S E L T E L K F L C L G R V G K R K
FLICE (1-34)  M D - F S R N L Y D I G E Q L D S E D L A S L K F L S L D Y I P Q R K
FLICE (101-132) - - - Y R V M L Y Q I S E E V S R S E L R S F K F L L Q E E I S K C K
PEA-15 (1-35) M V E Y G T L F Q D L T N N I T L E D L E Q L K S A C K E D T I P S E K

FADD (36-67) L E R V Q S G L D L L F S M L L E Q N D L E P G H T E L I R E L L - - -
FLICE (34-66) Q E P I K D A L M L F Q R L Q E K R M L E E S N L S F I K E L L F R I
FLICE (133-164) L D D D M N L L D I F I E M E K R V I L G E G K L D I L K R V C - - -
PEA-15 (36-67) S E E I T T G S A W F S F L E S H N K L D K D N L S I I E H I F - - -
    
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C



B

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L I I N N V N - - - - - F C R E S G L R T R T G S N I D C E K L R R R F P S S L H F M V E V - - K G D L T A K K M V L A ICE-LAP6
I I I N N K N - - - - - P D K V T G M G V R N G T D D K D A E A L F K C F R S L G F D V I V - - Y N D C S S C A K M Q D L ICE-LAP3
I I I N N K N - - - - - P H K S T G M T S R S S G T D V D A A N L R E T F R N L K Y E V R N - - K N D L T R E E I V E L Yama
I F N H E R F - - - - - F W H L T L E P E R R R T C A D R D N L T R R F S D L G F E V K C - - F N D L K A B E L L K Mch2α
L V L S N V H - - - - - F T G E K E L E P F R S G G D V D H S T L V T L F K L L G Y D V H V L C D Q - T A Q E M Q E K ICH1
L I I C N - - - - - E E F - - - D S I P R R T G A E V D I T G M T M L L Q N L G Y S V D V - - K K N L T A S D M T E hICE
L I I C N - - - - - T E F - - - D H L P P R N G A D F D I T G M K E L L E G L D Y S V D V - - E E N L T A R D M E S V ICE rel II
L I I C N - - - - - T K F - - - D H L P A R N G A H Y D I V G M K R L L Q G L G Y T V V D - - E K N L T A R D M E S V ICE rel III
L I I N N E H - - - - - F - - - E Q M P T R N G T K A D K A D N L T N L F R C M G Y T V - I C K D N L T G R G M L T CED-3
I I N N H F A K A R E K V P K L H S I R D R N G T H L D A G A L T T N T F F E E L H F E I K P - - H D D C T V E Q I Y E I FLICE

L L E L A R Q D - H G A L D C C V V V I L S H G C Q A S H L Q F P G A V Y G T D G C P V - S V E K I V N I F N G T S C P ICE-LAP6
L K K A S E E D - H T N A A C F A C I L L S H G E E - - - - - N V I Y G K D G - V T - P I K K D L T A H F R G D R C K ICE-LAP3
M R D V S K E D - H S K R S S F V C V L L S H G E E - - - - - G I I F G T N G - P V - D L K K I T N F F R G D R C R Yama
I H E V S T V S - H A D A D C F V C V P L S H G E G - - - - - N H I Y A Y D A - K I - E I Q T L T G L F K G D K C H Mch2α
L Q N F A Q L P A H R V T D S C I V A L L S H G V E - - - - - G A I Y G V D G K L L - Q L Q E V F Q L F D N A N C P ICH1
L E A F A R P E H K S S D S T F L V P M S H G I R E G - - I C G K K H S E Q V P D I L Q L N A I F N M L N T K N C P hICE
L R A F A T R P E H K S S D S T F L V L M S H G I L E G - - I C G T V H D E K K P D V L L Y D T I F Q I F N N R N C L ICE rel II
L R D F A A R P E H K S S D S T F L V L M S H G I L E G - - I C G T A H K K K K P D V L L Y D T I F Q I F N N R N C L ICE rel III
I R D F A K P E H S H - G D S A I L V I L S H G E - - - - - N V I I G V D I P I - S T H E I Y D L L N A A N A P CED-3
L K I Y Q L M D - H S N M D C F I C C I L S H G D R - - - - - G I I Y G T D G Q E P - P I Y E L T S Q P T G L K C P FLICE

S L G G K P K L F F I I Q A C G G E Q K D H G - - F E V A S T S P E D E S P G S N P E P D A T - P F Q E G L R T F D Q L ICE-LAP6
T L L E K P K L F F I I Q A C R G T E - - - - - L D D A I E Q A D S G P I N D T D A N P - R Y K - - - - - ICE-LAP3
S L T G K P K L F F I I Q A C R G T E - - - - - L D C G I E T D S G V D D M A C H - - - - - K - - - - - Yama
S L V G K P K I F I I I Q A C R G N Q H D V P V I P L D V V D N Q T E K L D T N I T E V D A A S - V Y T - - - - - Mch2α
S L Q N K P K M F F I I Q A C R G D E T D R G - - - - - V D Q Q D G K - - - - - N H A G S P - - - - - G C E E S D A G K ICH1
S L K D K P K V I I I I Q A C R G D S - - P G V V W F K D S V G V S G N L S L P T T E - - - - - E F hICE
S L K D K P K V I I V I Q A C R G E K A N - - R G E L W V R D S P A S L E V A S S Q S S E - - - - - N L ICE rel II
S L K D K P K V I I V I Q A C R G E K - - H G E L W V R D S P A S L A V I S S Q S S E - - - - - N L ICE rel III
R L A N K P K I V F V I Q A C R G E R R D N G F P V L D S V D G V P A P L R R G W D N R D G P L F N F L G C V R P Q V Q Q CED-3
S L A G K P K V F F I I Q A C O G D N Y Q K G - I P V E - - T D S E E Q P Y L E M D L S S P Q T - R Y - - - - - FLICE

D A I S - - S L T P T S D I F V S Y S T V P G F V S W R D P K S G S W Y V E T L D D I F E Q W - A H S E D L Q S L L R ICE-LAP6
- - - - - I P V E A D F L F A Y S T V P G Y Y S W R S P G R G S W F V Q A L C S I L E E H - G K D L E I M Q I L T R ICE-LAP3
- - - - - I P V D A D F L Y A Y S T A P G Y Y S W R N S K D G S W F I Q S L C A M L K Q Y - A D K L E F M H I L T R Yama
- - - - - L P A G A D F L M C Y S V A E G Y Y S H R E T V N G S W Y I Q D L C E M L G K Y - G S S L E F T E L L T L Mch2α
E K L P K M R L P T R S D M I C G Y A C L K G T A A M R N T K R G S W Y I E A L A Q V F S E R - G C D M H V A D M L V R ICH1
E D D A I K K A H I E K D F I A F C S S T P D N V S W R H P T M G S V F I G R L I E H M Q E Y - A C S C D V E E I F R K hICE
E E D A V Y K T H V E K D F I A F C S S T P H N V S W R D S T M G S I F I T Q L I T C F Q K Y - S W C C H L E E V F R K ICE rel II
E A D S V C K I H E E K D F I A F C S S T P H N V S W R D R T R G S I F I T E L I T C F Q K Y - S C C C H L M E I F R K ICE rel III
- - - - - V W R K K P S Q A D I L I R Y A T T A Q Y V S W R N S A R G S W F I Q A V C E V F S T H - A K D M D V V E L L T E CED-3
- - - - - I P D E A D F L L G M A T V N N C V S Y R N P A E G T W Y I Q S L W Q S L R E R C P R G D D I L T I L T E FLICE

V A N A V S V K G I Y - - - - - K Q M P G C F N F - L R K K L F F - - - - - K T S ICE-LAP6
V N D R V A R H P F E S Q S D D P H F H E K K Q I P C V V S M - L T K E L Y F - - - - - S Q ICE-LAP3
V N R K V A T E F E S P F S P D A T F H A K K Q I P C I V S M - L T K E L Y F Y - - - - - H Yama
V N R K V S Q R R V D F C K D P S A I G K K Q V P C F A S M - L T K K L H F E P - - - - - K S N Mch2α
V N A L I K D R - E G Y A P G T E P H R C K E M S E Y C S T - L C R H L Y L E P G - - H P P T ICH1
V R P S F E Q P - - - - - D G R A Q M P T T E R V T L T R C F Y L F P G - - - - - H hICE
V Q Q S F E T P - - - - - R A K A Q M P T I E R L S M T R Y F Y L F P G - - - - - N ICE rel II
V Q K S F E V P - - - - - Q A K A Q M P T I E R A T L T R D F Y L F P G - - - - - N ICE rel III
V N K - - K V A - C G F Q T S Q G S N I L K Q M P E M T S R - L L K K F Y F W P E A R N S A V CED-3
V N Y E V S N K - - - - - D D K K N M G K Q M P E P T F T - L R K K L V F - - - - - P S D FLICE
    
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Figure 3. FLICE Has Homology to Both FADD and ICE/CED3

- (A) Two domains contained within the N-terminal portion of FLICE (amino acids 1-34 and 101-132) were aligned with the N-terminus of FADD and PEA-15. Shading refers to identical residues.
- (B) The ICE/CED3 homologous domain of FLICE was aligned with other members of the ICE/CED-3 family. Shading refers to identical residues. The conserved pentapeptide motif is boxed. Based on the X-ray crystal structure of ICE, conserved residues involved in catalysis are indicated with filled circles; filled triangles represent the binding pocket for the carboxylate of the P1 Asp; filled squares indicate residues adjacent to the P2-P4 amino acids.
- (C) Phylogenetic analysis of ICE/CED-3 family members.

selective inhibitor of granzyme B (anti-GraB) as previously described (Quan et al., 1996). Importantly, both granzyme B-processed Yama and FLICE were active, as determined by their ability to cleave PARP (Figure 6B). Therefore, unlike ICE, FLICE and other members of the CED-3 subfamily are able to cleave PARP into signature apoptotic fragments (Tewari et al., 1995).

Overexpression of FLICE Induces Apoptosis, Which Is Abrogated by ICE Family Inhibitors
To study the functional role of FLICE, we transiently transfected MCF-7 breast carcinoma cells with an expression vector encoding the FLICE protein and subsequently assessed for apoptotic features. We chose MCF-7 cells since they are easily transfectable and are

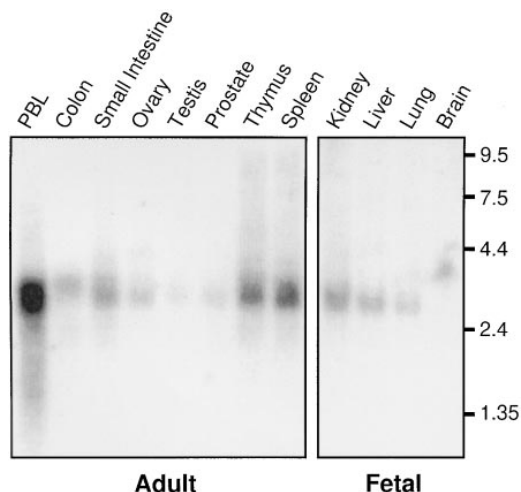


Figure 4. FLICE Is Expressed in a Variety of Tissues and Developmental Stages

A human adult and fetal tissue poly(A⁺) Northern blot (Clontech) was probed with ³²P-labeled FLICE cDNA. PBL, peripheral blood leukocytes.

sensitive to CD95-induced killing (Tewari and Dixit, 1995b). Like the other ICE/CED-3 family members, overexpression of FLICE caused apoptosis (Figure 7). The FLICE-transfected cells displayed morphological alterations typical of adherent cells undergoing apoptosis, becoming rounded, condensed, and detaching from the dish (data not shown). FLICE-induced cell death was inhibited by the broad spectrum ICE inhibitor z-VAD-fmk (Fearnhead et al., 1995; Pronk et al., 1996; Figure 7), which is also a potent inhibitor of CD95-induced apoptosis (A. M. C., unpublished data). Like the peptide ICE family inhibitor, CrmA blocked FLICE-induced cell death (Figure 7), suggesting that FLICE may be a physiologic target for this poxvirus serpin. Alternatively, FLICE may activate a downstream CrmA-sensitive protease.

Discussion

The Physical Link Between Death Receptors and Apoptotic Proteases

Prior to this study, the link between activators and effectors of the cell death machinery was unclear. Here we demonstrate that a cell-surface death receptor (CD95) uses an adaptor molecule (FADD) to engage physically a cytosolic apoptotic protease termed FLICE. The death domain is an important signaling motif shared by both CD95 and TNFR-1, and oligomerization of this domain recruits cytosolic adaptor proteins to assemble a signaling complex (DISC; Peter et al., 1996). The assembly of a DISC is essential for CD95 signal transduction. Upon activation, the death domain of the receptor binds to the death domain of the adaptor molecule FADD (CAP1, -2) and thereby recruits it to form part of the DISC. The complete DISC is created when FADD, in turn, binds and recruits the ICE/CED-3-like protease FLICE (CAP4).

While the main activity of CD95 is to trigger cell death, TNFR-1 can signal a diverse range of activities, including

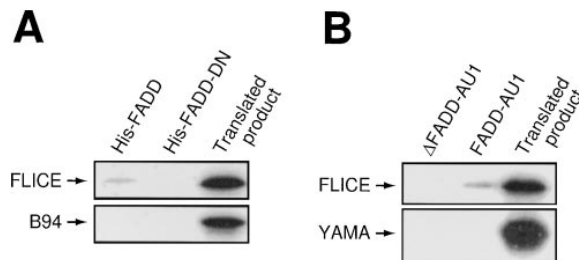


Figure 5. Specific Interaction of FADD with Radiolabeled FLICE

(A) Interaction of in vitro translated ³⁵S-labeled FLICE and a control protein, B94, with His-tagged FADD and His-tagged FADD-DN immobilized onto Ni²⁺ beads. After the beads were washed, retained bound FLICE or B94 proteins were analyzed by SDS-PAGE and autoradiography. The same amount of ³⁵S-labeled proteins used for the binding assay (input) was loaded directly onto the gel as a control. The gel was stained with Coomassie blue prior to autoradiography to confirm equivalent amounts of His-tagged FADD and FADD-DN in each lane (data not shown).

(B) Interaction of ³⁵S-labeled FLICE or Yama with FADD-AU1 or ΔFADD-AU1 lacking only 18 N-terminal residues. 293T cells were transfected with AU1 epitope-tagged FADD or AU1 epitope-tagged ΔFADD and the cleared lysates immunoprecipitated with anti-AU1 antibody and protein G-coupled Sepharose. Following incubation with in vitro translated ³⁵S-labeled FLICE or Yama, the beads were washed and retained FLICE or Yama detected by SDS-PAGE and autoradiography. The presence of equivalent amounts of FADD-AU1 and ΔFADD-AU1 in the immune complex was assessed by immunoblotting using a specific rabbit antiserum raised against FADD (data not shown).

fibroblast proliferation, resistance to intracellular pathogens including chlamidia, and synthesis of prostaglandin E2 (Tartaglia and Goeddel, 1992). Likewise, TNFR-1 recruits a multivalent adaptor molecule termed TRADD, which, like FADD, contains a death domain required for receptor association (Hsu et al., 1995). TRADD has been shown to bind a number of signaling molecules, including FADD, TNFR-associated protein 2, and RIP (Hsu et al., 1996a, 1996b). A dominant-negative version of FADD blocks TNF killing (Chinnaiyan et al., 1996a; Hsu et al., 1996a), while dominant-negative versions of TNFR-associated protein 2 and RIP block TNF-induced NF-κB activation (Hsu et al., 1996a, 1996b). This indicates the existence of a signaling bifurcation dictated by the nature of the associated adaptor molecules. Regardless, TNFR-1 indirectly uses FADD to engage the death protease FLICE and thereby unites the death pathways that emanate from CD95 and TNFR-1.

Emphasizing the evolutionary conservation of the apoptotic machinery, the *Drosophila* death protein Reaper shares weak homology to the death-domain motif (Golstein et al., 1995) and, like CD95 and TNFR-1, is capable of initiating the apoptotic program (White et al., 1994, 1996). Reaper mediates its actions by activating ICE/CED-3-like proteases as both z-VAD-fmk and the baculovirus ICE family inhibitor p35 block Reaper-induced cell death (Pronk et al., 1996; White et al., 1996). Given our data on death-domain receptors, it is tempting to speculate that Reaper may exert its actions by directly or indirectly engaging ICE/CED-3-like proteases in a manner analogous to FADD and CD95, respectively.

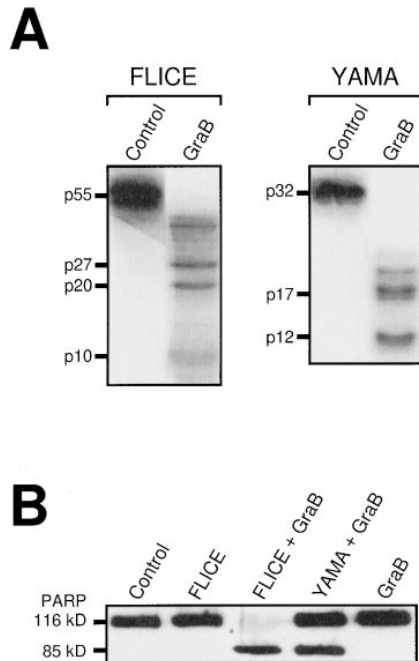


Figure 6. Granzyme B-Processed FLICE Is an Active Protease That Cleaves PARP to the Signature 85 kDa Apoptotic Fragment

(A) Processing of FLICE and Yama by granzyme B (GraB) in vitro. ³⁵S-labeled FLICE and Yama were incubated with purified GraB for 4 hr and the reaction products analyzed by SDS-PAGE and autoradiography. The active subunits of Yama (p17/p12) and FLICE (p20/p10) are indicated. A 27 kDa species of FLICE was also generated by granzyme B, likely representing the FLICE prodomain. (B) GraB-processed FLICE and Yama cleave PARP. Following GraB processing, anti-GraB, a specific GraB inhibitor, was added and proteolytically processed FLICE or Yama assessed for activation by incubating with the putative substrate PARP. No cleavage was detectable when PARP was incubated alone (control) or with the zymogen-unprocessed form of FLICE. PARP was detected by immunoblotting using a specific anti-PARP antibody.

FLICE Is Homologous to FADD and Is a Cysteine Protease of the ICE/CED-3 Family

FLICE is an intriguing molecule with homology to both FADD and the ICE/CED-3 family. The prodomain of FLICE is homologous to the DED of FADD, which is a critical motif required for engaging the death pathway. Phylogenetic comparison of FLICE with other ICE/CED-3 family members classify this cysteine protease in the CED-3 subfamily along with Yama/CPP-32, ICE-LAP3, ICE-LAP6, and Mch-2 (Figure 3C). FLICE clearly represents the most upstream enzymatic activity in the CD95 pathway, and it will thus be important to determine which ICE/CED-3 family members are proteolytically activated by FLICE, leading to amplification of the death signal. Though activated FLICE cleaves PARP well (Figure 6), this is unlikely to be the physiologic substrate for FLICE and was only used as a readout to monitor the generation of proteolytically competent FLICE by granzyme B (Figure 6). Physiologic PARP cleavage is more likely mediated by the downstream enzymes Yama and ICE-LAP3 (Fernandes-Alnemri et al., 1995; Lippke et al., 1996; Nicholson et al., 1995; Tewari et al., 1995).

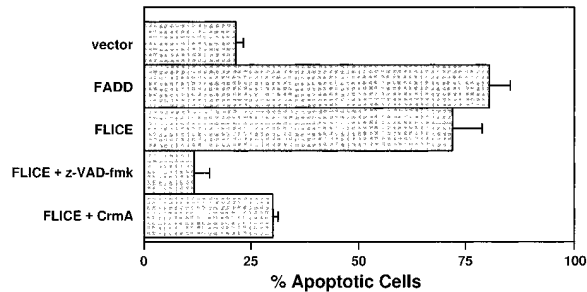


Figure 7. FLICE-Induced Apoptosis Is Blocked by ICE/CED-3 Inhibitors z-VAD-fmk and CrmA

Previously characterized MCF-7 clonal lines (Tewari et al., 1995a) were transiently transfected with pCMV- β -galactosidase in the presence of a two-fold excess of vector alone or expression constructs encoding FADD or FLICE. The broad spectrum ICE family inhibitor z-VAD-fmk (20 μ M) was added to the cells 5 hr posttransfection. Cells were fixed and stained for β -Gal expression 42 hr following transfection. The data (mean plus or minus standard error of the mean) shown are the percentage of round blue cells as a function of the total number of blue cells counted (n = 3).

Importantly, the Asp-specific proapoptotic serine protease granzyme B proteolytically activates FLICE in a manner similar to Yama/CPP-32 (Darmon et al., 1995; Quan et al., 1996). By contrast, ICE is processed, but not activated, by granzyme B (Quan et al., 1996). Secretion of granzyme B, in combination with the pore-forming protein perforin, is one of two mechanisms by which cytotoxic T lymphocytes trigger apoptosis of susceptible target cells, the other being engagement of CD95 on the target cell surface (Berke, 1995). Thus, granzyme B likely mediates apoptosis by directly activating the death effector machinery of the target cell, which is composed of an arsenal of intracellular CED-3-like cysteine proteases. FLICE and other members of the CED-3 subfamily (K. Orth and A. M. C., unpublished data) are almost certainly activated during both granzyme B- and CD95-mediated apoptosis, suggesting the convergence of distinct apoptotic pathways involved in cell-mediated immunity.

Identity of CAP3

CAP3 likely represents the N-terminal prodomain of FLICE, consistent with the finding that the predicted molecular mass and pI of the N-terminal 220 amino acids of FLICE correspond to CAP3. Like CAP4, CAP3 was only found to be associated with the activated CD95 receptor requiring full-length FADD for binding (Chinnaiyan et al., 1996a). It is conceivable that CAP3 is generated by proteolytic activation of FLICE during CD95 ligation but retained in the DISC by virtue of its ability still to bind the DED of FADD. The active dimeric p20/p10 subunits of FLICE are likely liberated from the DISC and are free to cleave and activate downstream apoptotic proteases. If this scenario is correct, CAP4 represents the inactive zymogen form of FLICE, which during CD95 signaling gets activated, generating a free active heterodimeric enzyme and a prodomain that is still retained by the DISC (CAP3).

How Is FLICE Activated?

Taken together, our data enable us to propose an intriguing model for FLICE activation. In the latent state, the two DEDs of FLICE may bind to each other, preventing FLICE activation. CD95 stimulation and resultant trimerization causes binding of FADD. This may trigger a conformational change in the DED of FADD, allowing it to bind one of the corresponding DEDs of FLICE. By disrupting the self-association of the FLICE DEDs, the ICE/CED-3 homology domain of FLICE may be free to undergo autocatalytic activation. The active p20/p10 protease is liberated from the DISC and subsequently ignites a proteolytic cascade composed of other ICE/CED-3 family members. The FLICE prodomain (CAP3) remains bound to the receptor.

Nano-ES MS/MS as a Technique to Identify Protein Interaction Partners

The cloning of FLICE represents a unique instance of a protein being identified by direct sequencing of a silver-stained spot (containing protein in the femtomole range) from a 2D isoelectric focusing (IEF)/SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel by nano-ES MS/MS. The exceptional sensitivity of this technique opens up new experimental vistas. Just as it did in this report, it will allow investigators to analyze endogenous components of signaling complexes, confirming and extending findings obtained from the application of techniques such as the two-hybrid system, which, in particular, is plagued by false positive interactions. Additionally, nano-ES MS/MS can be used to identify proteolytic cleavage products (such as in the case of CAP3) and sites of posttranslational modifications including phosphorylation. As more cDNA sequences accumulate, the technique should become generally applicable to most human gene products.

Conclusions

The CD95-mediated apoptotic cascade is initiated by the direct physical association of the death receptor CD95 with the adaptor molecule FADD and the effector protease FLICE, a novel member of the ICE/CED-3 family. The assembly of this signaling complex occurs in a hierarchical manner upon receptor activation. The death domain of the receptor binds to the death domain of the adaptor molecule FADD, which, in a manner that remains to be determined, binds and activates FLICE to generate the most apical enzymatic component of a death cascade composed of other ICE/CED-3 family members. The essential components of an apoptotic cascade have here been elucidated. The mode of assembly of the DISC and the mechanism by which FLICE is activated will be critical questions for future investigation.

Experimental Procedures

Preparation of CAP3 and CAP4 for Sequencing

The human full-length CD95 transfectant K50 (Oehm et al., 1992) was cultured in 5 l of RPMI with 10% fetal calf serum, 10 mM HEPES (pH 7.3), 100 μ g/ml Gentamycin to a density of 2.8×10^6 cells/ml. Cells (1.4×10^{10}) were then stimulated in 100 ml medium with 10 μ g/ml anti-APO-1 (IgG 3) for 10 min at 37°C. After washing once with phosphate-buffered saline, the cells were lysed on ice for 30

min with 50 ml lysis buffer (20 mM Tris-HCl [pH 7.4], 140 mM NaCl, 10% [w/v] glycerol, 1% [v/v] Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μ g/ml of the following peptide inhibitors: antipain, chymostatin, leupeptin, and pepstatin A). After two centrifugations for 15 min at 14,000 rpm, the supernatant was pre-cleared with 400 μ g of the isotype-matched control antibody FII23c (Kischkel et al., 1995) coupled to 400 μ l of CNBr-activated Sepharose CL-4B (Pharmacia) for 12 hr. The DISC was then immunoprecipitated with 200 μ l of a 50% suspension of PA-Sepharose (Pharmacia). After 12 hr, the beads were washed five times with lysis buffer and the bound proteins solubilized in IEF sample buffer, then subjected to IEF essentially as described (Peter et al., 1995). The IEF sample buffer contained 9.8 M urea, 4% (w/v) NP-40, 2% (v/v) ampholines (pH 7.9; Pharmacia) and 100 mM dithiothreitol. The tube gel used for the first dimension was 25 cm long and had an internal diameter of 2.5 mm. The IEF gel was run at 1200 V for 20 hr. For the second dimension, a 200 \times 1 mm 10% SDS-PAGE gel was used. Protein spots were visualized by silver staining as described (Shevchenko et al., 1996). The identities of silver-stained CAP3 and CAP4 with the respective ³⁵S-labeled CAP4 spot described recently (Kischkel et al., 1995) was confirmed in a parallel experiment in which unlabeled and labeled protein were separated on the same gel, which was first silver-stained, then dried and subjected to autoradiography.

Sequencing of CAP3 and CAP4 with Nano-ES MS/MS

The silver-stained CAP3 and CAP4 spots were cut out and in-gel-digested with trypsin, followed by a single purification and concentration step as previously described (Shevchenko et al., 1996; Wilm et al., 1996). The recovered unseparated peptide mixtures were sequenced on a triple quadrupole mass spectrometer API III (Sciex) equipped with a nano-electrospray ion source (Wilm and Mann, 1996).

cDNA Cloning

The sequence obtained from the characterized peptides was used to screen the Human Genome Sciences private database using established expressed-sequence-tag methods. A clone designated HHHFY89 was identified and subjected to both automated and manual sequencing. Additionally, a random-primed cDNA library constructed in the pcDNA1 vector (Invitrogen) from human umbilical vein endothelial cell poly(A)⁺ RNA was screened with a ³²P-labeled BamHI-XhoI restriction fragment of the HHHFY89 clone (Sambrook, 1989). DNA sequence analysis of hybridizing clones confirmed the original sequence.

Northern Blot Analysis

Adult and fetal human multiple tissue Northern blots (Clontech) were hybridized according to instructions of the manufacturer, using a radiolabeled cDNA insert obtained from a BamHI-XhoI digestion of the HHHFY89 plasmid.

Expression Vectors

FLICE cDNA encoding the entire open reading frame was generated by polymerase chain reaction and subcloned into the mammalian expression vector pcDNA3 (Invitrogen).

His-tagged FADD and His-tagged FADD-DN were obtained using custom polymerase chain reaction primers encoding the epitope and FADD cDNA as template. The amplified products were sub-cloned into the prokaryotic pET vector system (Novagen). FADD-AU1 and the truncated form of FADD lacking 18 N-terminal amino acids (designated Δ FADD-AU1) were similarly generated using custom polymerase chain reaction primers encoding the epitope and FADD cDNA as template. Polymerase chain reaction products were subcloned into the KpnI-XhoI sites in the pcDNA3 expression vector.

In Vitro Binding Assay

His-tagged FADD and His-tagged FADD-DN proteins were expressed, purified, and immobilized onto Ni²⁺ beads according to standard methodology.

³⁵S-labeled FLICE, Yama, and B94 proteins were prepared by in vitro transcription or translation using the TNT T7-coupled reticulocyte lysate system (Promega) according to the instructions of the

manufacturer, using cDNAs encoding either FLICE, Yama, or B94 as template.

³⁵S-labeled FLICE or B94 was diluted in binding buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 1% NP-40) and incubated for 3 hr at 4°C with the various His-tagged proteins immobilized onto Ni²⁺ beads; following recovery of the beads by pulse centrifugation, they were washed three times with binding buffer, boiled in SDS sample buffer, and resolved on a 12.5% SDS-polyacrylamide gel. Bound proteins were visualized following autoradiography.

Alternatively, 293T cells, a human epithelial cell line constitutively expressing the SV-40 large T antigen, were transfected as previously described (O'Rourke et al., 1992) with either FADD-AU1 or ΔFADD-AU1 expression constructs and, 36 hr later, the cells lysed in binding buffer, adjusted to 500 mM NaCl, and immunoprecipitated with anti-AU1 antibody and protein G-coupled Sepharose. Immobilized FADD protein was incubated with equivalent amounts of ³⁵S-labeled FLICE or YAMA and incubated for 3 hr at 4°C, washed three times with binding buffer, boiled in SDS sample buffer, and resolved on a 12.5% SDS-polyacrylamide gel. FADD-AU1 and ΔFADD-AU1 proteins were visualized by immunoblotting, using a rabbit polyclonal antiserum directed against FADD essentially as described previously (Chinnaiyan et al., 1996a).

Activation of FLICE and In Vitro Reconstitution Experiments

³⁵S-labeled FLICE was activated by incubating at 37°C for 4 hr with 1 pmol of granzyme B in reaction buffer (50 mM HEPES [pH 7.4], 0.1% CHAPS, 0.1 M NaCl, 10% sucrose) supplemented with dithiothreitol (10 mM), as described previously (Quan et al., 1996). Following activation, a specific recombinant-engineered serpin inhibitor of granzyme B (anti-GraB) was added and the reaction incubated at 37°C for an additional 15 min. The putative substrate PARP (150 ng) was then added and the reaction allowed to proceed for 2 hr at 37°C. A control reaction containing PARP and the proform of FLICE was carried out in parallel, except that no granzyme B was added during the procedure.

Cell Death Assay

MCF-7 human breast carcinoma clonal cell lines stably transfected with either vector alone or a CrmA expression construct (Tewari and Dixit, 1995b) were transiently transfected with pCMV-β-galactosidase in the presence of a four-fold excess of pcDNA3 expression constructs encoding either FADD or FLICE (Chinnaiyan et al., 1995). The ICE family inhibitor z-VAD-fmk (Enzyme Systems Products) was added to the cells at a concentration of 20 mM, 5 hr after transfection. Following transfection (42 hr), cells were fixed and stained with X-Gal as previously described (Chinnaiyan et al., 1995). The data (mean plus or minus standard error of the mean) shown are the percentage of round blue cells among the total number of blue cells counted. Data were obtained from at least three independent experiments.

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